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Exploiting species differences to understand the CFTR Cl^- channel

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ABSTRACT

The anion channel cystic fibrosis transmembrane conductance regulator (CFTR) is a unique ATP-binding cassette (ABC) transporter. CFTR plays a pivotal role in transepithelial ion transport as its dysfunction in the genetic disease cystic fibrosis (CF) dramatically demonstrates. Phylogenetic analysis suggests that CFTR first appeared in aquatic vertebrates fulfilling important roles in osmosensing and organ development. Here, we review selectively knowledge of CFTR structure, function and pharmacology, gleaned from cross-species comparative studies of recombinant CFTR proteins, including CFTR chimeras. The data argue that subtle changes in CFTR structure can impact strongly on channel function and the action of CF mutations.

ABBREVIATIONS

ABC transporter, ATP-binding cassette transporter; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ECL, extracellular loop; ICL, intracellular loop; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; RD, regulatory domain

INTRODUCTION

The life-shortening genetic disease cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. CFTR is a unique member of the ATP-binding cassette (ABC) transporter superfamily (ABCC7) that functions as an anion-selective channel [2]. CFTR is found primarily in the apical membrane of epithelial cells lining ducts and tubes, where it plays a key role in epithelial fluid and electrolyte transport and the maintenance of cellular homeostasis [3]. Loss of CFTR function in CF causes the blockage of ducts and tubes by thick, tenacious mucus, leading to the development of diverse organ pathologies (e.g. chronic lung disease, exocrine pancreatic failure, meconium ileus (blockage of the intestine in neonates), male infertility and salty sweat) [3,4].

To date, more than 2,000 CF-associated mutations have been identified within the CFTR gene (http://www.genet.sickkids.on.ca/app). As summarised in Figure 1A, CF-associated mutations are assigned to one of six different classes according to their impact on CFTR production, processing, plasma membrane stability and channel function (for review, see Ref [5]). While some CF-associated mutations disrupt CFTR function by a single mechanism, frequently they have complex effects on CFTR expression and function. For example, the most common CF mutant, F508del-CFTR is assigned to class II (defective processing), but it also exhibits defects characteristic of classes III (defective channel regulation) and VI (reduced protein stability) [5]. This highlights the challenge of elucidating mechanisms of dysfunction to inform the development of mutation-specific therapies for CF.

Current therapy for CF treats disease symptoms. To date, the only mutation-specific therapy approved for patient use is ivacaftor, a small molecule that potentiates CFTR channel gating, developed by Vertex Pharmaceuticals (Boston, USA) with support from Cystic Fibrosis Foundation (Bethesda, USA) [6,7]. However, use of ivacaftor is restricted to a small cohort of CF patients carrying class III mutations affecting CFTR channel gating (e.g. G551D-CFTR [8,9]) [4]. In addition to potentiation of channel gating, mutation-specific therapy for F508del-CFTR must correct defective protein delivery to and stability at the plasma membrane [5,10]. Clinical trials of combination therapy (ivacaftor and lumacaftor (Vertex Pharmaceuticals), a small molecule that corrects the F508del-CFTR processing defect) were completed in 2014 and are now being evaluated for regulatory approval [11]. Because the F508del mutation affects CFTR folding in multiple ways (e.g. [12,13]), combinations of CFTR correctors will likely be required to fully restore CFTR function.

One barrier to the development of mutation-specific therapies for CF is the lack of an atomic-resolution structure of the entire CFTR protein. In its absence, molecular models of CFTR have been constructed using the crystal structures of related ABC transporters (e.g. Sav1866 [14]) as templates [15,16]. Figure 1B shows CFTR in a nucleotide-bound outward-facing configuration. In this configuration, the nucleotide-binding domains (NBDs) form a head-to-tail dimer stabilised by ATP binding to two ATP-binding sites (site 1, non-hydrolytic; site 2, hydrolytic) located at the dimer interface. By contrast, the membrane-spanning domains (MSDs) form an asymmetric hour-glass with a deep wide intracellular vestibule and a shallow wide extracellular vestibule separated by a constriction located towards the extracellular end of the pore. Functional evidence argues that the pore constriction not only determines the anion selectivity of CFTR [17], which follows a lyotropic series [18], but is also the location of a gate [19]. Of note, this gate location supports the degraded ABC transporter model of CFTR function [20], which argues that CFTR evolved from a double-gated ancestral ABC transporter by atrophy of one gate. Communication between the NBDs and MSDs is mediated by four coupling helices, located at the tips of four intracellular loops (ICLs), long α-helical extensions

of transmembrane segments in MSD1 and MSD2 [15,16]. A unique feature of CFTR is its regulatory domain (RD) (Fig. 1B), an intrinsically disordered protein segment, distinguished by multiple consensus phosphorylation sites, which interacts with all intracellular regions of CFTR [21]. RD phosphorylation not only relieves steric inhibition of NBD1:NBD2 dimerisation by the RD, but also stimulates the interaction of ATP with the ATP-binding sites (for review, see Ref [21]). ATP binding and hydrolysis at site 2 then drives cycles of NBD1:NBD2 dimer assembly and disassembly [2]. Movement of the NBD1:NBD2 dimer initiates a wave of conformational change, which moves through the ICLs to the MSDs to gate the pore constriction and hence, control anion flow [22].

EMERGENCE OF CFTR: A Cl^- CHANNEL FOR OSMOSENSING AND ORGAN DEVELOPMENT

Following the identification of CFTR and the cloning of its cDNA [1], CFTR homologues have been discovered in a diverse range of vertebrates (Figure 2 and Supplementary Material Table 1) [23]. It is unclear when CFTR first appeared within the phylogenetic tree. However, molecular evolution studies, which exploit the RD as a unique feature of CFTR, argue that CFTR emerged early in the development of gnathostomes (jawed vertebrates) and is absent among other chordate classes (e.g. lamprey and hagfish) [24]. Interestingly, these studies argue that the RD originated from non-coding intronic sequences, providing an explanation for its intrinsically disordered structure [24]. Of note, no CFTR homologue has been confirmed in the model invertebrate organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, in contrast to sulphonylurea receptors and multidrug resistance-associated proteins [25,26], both close relatives of CFTR in the C subdivision of the ABC transporter superfamily. The identification of CFTR expression in teleost fish such as *Takifugu rubripes* [27], *Fundulus heteroclitus* (killifish) [28] and two distinct homologues in the tetraploid Atlantic Salmon *Salmo salar* [23] suggests that CFTR might have evolved in aquatic vertebrates to play a key role in osmoregulation. Indeed, studies of mitochondrion-rich cells in the gills and opercular epithelium of killifish demonstrate that CFTR is the apical membrane Cl^- channel in these osmosensing ion transporting epithelia [29]. Interestingly, in mitochondrion-rich cells of killifish, the tyrosine kinase focal adhesion kinase integrates both cAMP-dependent and osmotically-mediated cAMP-independent intracellular signals to regulate CFTR [30]. Changes in osmolarity exert complex effects on CFTR expression and function, initially altering CFTR regulation and protein recycling at the apical membrane of mitochondrion-rich cells, but later modifying gene expression [30,31].

While other anion channels also likely play a role in morphogenesis, studies of the model organism *Danio rerio* (zebrafish) suggest an important role for CFTR in organ development by driving lumen expansion during the formation of ducts and tubes [32]. For example, loss of Cse1l (chromosome segregation 1-like), a negative regulator of CFTR-mediated transepithelial ion transport, leads to a striking expansion of the intestinal lumen in zebrafish larvae [33]. Conversely, CFTR knockout in zebrafish embryos prevents lumen formation in Kupffer's vesicle, which specifies the left-right body axis [34]. The absence of left-right symmetry defects in CF patients argues that CFTR, itself, does not determine organ location in humans. Nevertheless, there is some evidence for developmental defects in CF (e.g. tracheal narrowing caused by altered smooth muscle function [35]). While CFTR loss does not prevent lumen formation in zebrafish pancreas [36], the role of CFTR in duct and tube formation, particularly the vas deferens, the duct most sensitive to CFTR loss [37], should be examined further.

SPECIES DIFFERENCES IN CFTR CONDUCTANCE, GATING AND PHARMACOLOGY

To date, the single-channel behaviour of only a limited number of recombinant CFTR homologues has been investigated (spiny dogfish [shark], [38]; frog [39]; mouse, [40]; chicken, [41]; bushtail possum, [42]; rabbit, [41]; sheep, [43]; pig, [44]). In all cases, expression of recombinant CFTR generated Cl^--selective channels regulated by cAMP-dependent phosphorylation and intracellular ATP. However, frequently studies of CFTR homologues have revealed surprising differences in single-channel behaviour, which inform understanding of structure-function relationships. Here, we selectively review the literature.

Shortly after the identification of the CFTR gene, mouse models of CF emerged, which highlighted interspecies differences in disease phenotype (for review, see Ref [45]). Amino acid sequence analysis reveals that murine CFTR differs noticeably from human CFTR, possessing an amino acid sequence identity similar to that of *Xenopus* CFTR (Fig. 2 and SM Table 1). The first single-channel recordings of murine CFTR suggested that its channel activity is greatly reduced compared to that of human CFTR [40]. These recordings identified the reduced single-channel current amplitude of murine CFTR and the short-lived sojourns to its full open-state (Fig. 3A, left and B). However, they failed to detect a tiny, long lived subconductance state of murine CFTR, which was only detected following inconsistencies between iodide efflux and single-channel studies of CFTR modulators and required heavy filtering for its resolution [46] (Fig. 3A, right). Thus, the gating behaviour of murine CFTR is characterised by prolonged openings to a tiny subconductance state (O_1) and only brief transits to the full open state (O_2) [46] (Fig. 3A). As a result, the open probability of murine O_1 is greater than that of human CFTR, whereas that of murine O_2 is dramatically reduced [46] (Fig. 3C).

Interestingly, AMP-PNP and pyrophosphate, two agents that potentiate robustly human CFTR channel gating do not augment the activity of murine CFTR [40]. Consistent with these data, a number of potentiators (e.g. VRT-532, NPPB-AM and ivacaftor) which augment robustly human CFTR channel gating have little or no effect on murine CFTR [6,47]. While these results argue that CF mice are unsuitable for testing CFTR potentiators, this is not the case for CFTR correctors. For example, the off-patent drug glafenine improved the trafficking of murine F508del-CFTR *ex vivo* and partially restored salivary secretion *in vivo* in CF mice with the F508del mutation, without causing adverse effects [48]. These data argue that CF mice have a role to play in evaluating rational new therapies for CF.

Species-dependent differences in CFTR inhibition by open-channel blockers and allosteric inhibitors have also been identified. First, shark CFTR is insensitive to the thiazolidinone $CFTR_{inh}$-172, an allosteric inhibitor that modulates channel gating [49]. Second, porcine CFTR is unaffected by the sulphonylurea drug glibenclamide, which occludes the intracellular vestibule of the CFTR pore [49]. Third, murine CFTR is insensitive to the glycine hydrazide GlyH-101, which obstructs the extracellular vestibule of the CFTR pore [50]. To explain these differences in channel inhibition, Stahl *et al*. [49] postulated that subtle differences in the three-dimensional structure of the CFTR pore and the local environment (e.g. hydrophobicity and charge) in the vicinity of drug-binding sites might be responsible. In future studies, these differences might be exploited to identify drug-binding sites on CFTR.

Towards the development of a sheep model of CF, we recently investigated with Ann Harris (Northwestern University Feinberg School of Medicine) the single-channel behaviour of ovine CFTR, a CFTR homologue with a high degree of sequence identity to human CFTR (Fig.

2 and SM Table 1). As demonstrated in Figure 3, ovine CFTR is noticeably more active than human CFTR. The reasons are three-fold: (i) the single-channel conductance of ovine CFTR is greater than that of human CFTR; (ii) the open probability of ovine CFTR is larger than that of human CFTR and (iii) ATP more strongly stimulates ovine CFTR channel gating [43]. For human CFTR, ATP predominantly regulates channel gating by increasing the frequency of channel openings [2]. However, in ovine CFTR, ATP accelerates the rate of channel opening, while simultaneously slowing the rate of channel closure [43]. Based on the energetic coupling model of CFTR channel gating [51], one possible explanation for the single-channel behaviour of ovine CFTR is that its gating cycle might be uncoupled from its ATP hydrolysis cycle. With Isabelle Callebaut (IMPMC, Sorbonne Universités), we sought structural explanations for the single-channel behaviour of ovine CFTR using Sav1866-based molecular models of CFTR [16] (Fig. 1B). This analysis revealed no clear differences between human and ovine CFTR within critical motifs in the MSDs and NBDs [43]. Instead, amino acid sequence differences were mostly confined to regions with known (or predicted) regulatory roles and characterised by the presence of additional serine and threonine residues in ovine CFTR [43]. In the future, it will be interesting to explore the regulation of ovine CFTR by protein kinases and phosphatases.

Variation in experimental conditions hampers data comparison between different studies of CFTR homologues. Nevertheless, data suggest that single-channel conductance varies between species, decreasing in the rank order chicken >> ovine ≥ rabbit ≥ *Xenopus* > human ≥ possum > porcine > murine ≥ shark [38-44,52]. Figure 3D explores the relationship between single-channel conductance and mean body temperature for endotherms. These data raise the interesting possibility that metabolic activity might influence CFTR behaviour. Future studies should investigate this possibility by studying CFTR homologues under identical experimental conditions.

SPECIES DIFFERENCES AS A TOOL TO UNDERSTAND CFTR STRUCTURE AND FUNCTION

While site-directed mutations are the method of choice to explore structure-function relationships, studies of CFTR chimeras have an important role to play. For example, studies of human-*Xenopus* CFTR chimeras provided early evidence that the first extracellular loop (ECL1) is an important determinant of CFTR channel gating. Single-channel studies revealed that the chimera hX1-6 containing MSD1 of *Xenopus* CFTR on a human CFTR background had an unusual pattern of channel gating distinct from the bursting pattern exhibited by human and *Xenopus* CFTR [39]. Recognising that the gating pattern of hX1-6 resembled the CF mutant R117H-CFTR [53], Price *et al*. [39] focused on sequence differences in the ECLs. Substitution of human ECL1, but not human ECL3, restored the bursting pattern of channel gating to hX1-6 [39]. Interestingly, guided by a molecular model of CFTR, Cui *et al*. [54] demonstrated that three charged residues in ECL1 (D110, E116 and R117), conserved across species, stabilise the conformation of the CFTR pore by forming salt bridges with nearby charged residues in the extracellular vestibule.

Using homologous recombination to generate a library of human-murine CFTR chimeras exchanging human NBD and RD sequences with the corresponding regions of murine CFTR, we demonstrated that NBD1 and NBD2 together determine the prolonged duration of the subconductance state of murine CFTR [55]. Because human-murine CFTR chimeras with one human and one murine NBD were functional [55], channel gating, albeit suboptimal, is possible when NBDs from two homologues are mixed. Moreover, studies of the processing of human-murine CFTR chimeras demonstrated that with two exceptions, replacement of human sequences with their murine equivalents had no adverse effects on CFTR folding [56]. Taken

together the data raise the interesting possibility of using chimeric channels with CFTR's MSDs to investigate the gating cycle of NBDs from other ABC transporters.

SPECIES DIFFERENCES AS A TOOL TO INVESTIGATE CF-ASSOCIATED MUTATIONS

In marked contrast to its impact on human CFTR, the F508del mutation has variable effects on the processing of CFTR homologues ranging from less severe in mammalian homologues (e.g. porcine and murine) to almost no effect in non-mammalian species (e.g. chicken and frog) [41,44]. Moreover, F508del chicken CFTR exhibits marked thermostability, remaining active at physiological temperatures, unlike F508del human CFTR, which deactivates promptly [41]. Hypothesizing that structural differences between human and chicken CFTR account for the thermostability of F508del chicken CFTR, Aleksandrov *et al*. [41] demonstrated that the F508del revertant I539T and four proline residues at key positions within NBD1 (S422P, S434P, S492P and A534P) were responsible for rescuing the processing, plasma membrane stability and function of human CFTR. These data argue that correction of NBD1 structure is sufficient to suppress fully the deleterious effects of the F508del mutation on human CFTR. However, Ostedgaard *et al*. [44] interpreted species-dependent effects of the F508del mutation on CFTR processing and channel function to suggest that these defects have different causes. In support of this idea, studies of human-murine CFTR chimeras revealed that maturation of F508del-CFTR requires NBD1, whereas rescue of F508del-CFTR channel gating necessitates NBD1-MSD2 interactions [57]. Consistent with the ideas of Ostedgaard *et al*. [44], other studies (e.g. [12,13]) demonstrate that correction of NBD1 folding and the interaction of NBD1 with ICL4 are required to suppress fully the defective protein processing and channel function of F508del-CFTR.

CONCLUSIONS

Cross-species comparative studies are a powerful approach to investigate the physiological role of CFTR and its dysfunction in disease. They have the potential to reveal surprising differences in channel function despite high degrees of shared amino acid sequence identity. Testing small molecules on heterologously expressed CFTR homologues informs proof of principle studies in animal models of disease, while chimeric CFTR constructs provide exciting opportunities to identify drug-binding sites on CFTR. Future studies should confront the challenge of investigating the single-channel properties of endogenous CFTR Cl^- channels in native epithelial cells. Ultimately, such studies in humans have the potential to illuminate genotype-phenotype-CFTR function relationships. We should not shy from this challenge.

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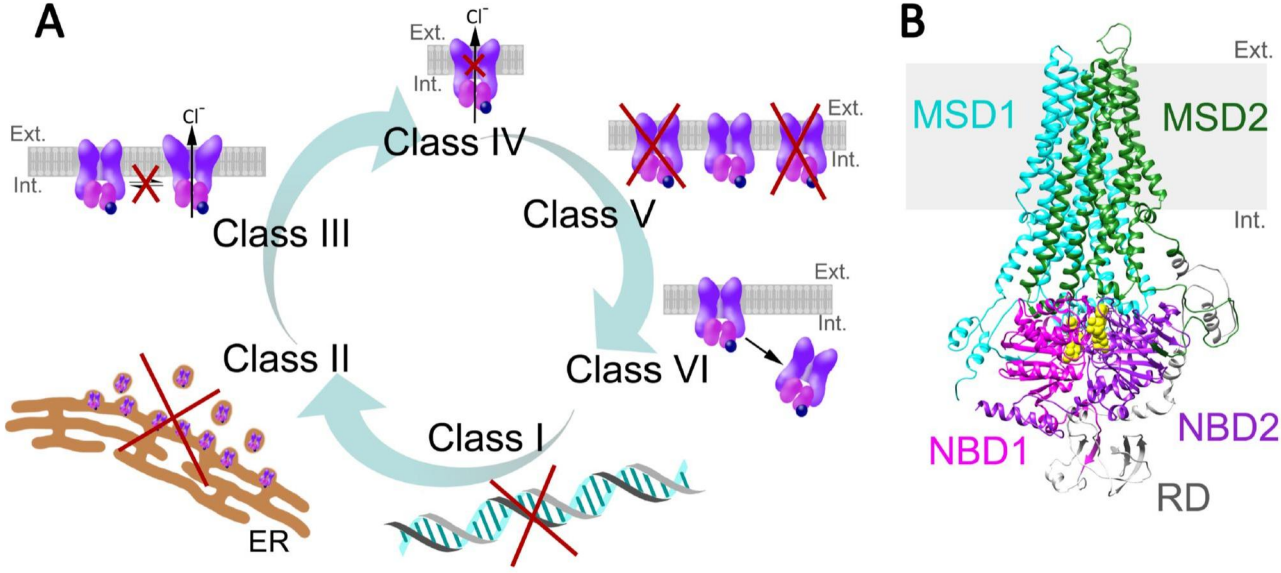
FIGURE LEGENDS

Figure 1: Molecular mechanisms of CFTR dysfunction in CF (**A**) Classification of CF-associated mutations based on their effects on CFTR production, maturation, plasma membrane stability and function. Class I mutations, defective protein production; class II mutations, defective protein processing; class III mutations, defective channel regulation; class IV mutations, defective channel conduction; class V mutations, reduced protein synthesis and class VI mutations, reduced protein stability. (**B**) Homology model of CFTR based upon *Staphylococcus aureus* Sav1866 [14] in an ATP-bound outward-facing configuration [58]. Membrane-spanning domain 1 (MSD1) is coloured cyan; MSD2, green, NBD1, violet; NBD2, purple; RD, grey and ATP molecules, yellow. The position of the plasma membrane is indicated with Int. and Ext. denoting the intra- and extracellular sides of the membrane, respectively. Reproduced, with permission, from Ref [58].

Figure 2: Phylogenetic relationship of CFTR The cladogram was constructed following ClustalO alignment of experimentally-derived and predicted whole-protein CFTR amino acid sequences. With the exception of mouse and rat [59], this cladogram largely reflects the evolutionary tree of the listed species. For further information, see Supplementary Material Table 1.

Figure 3: The single-channel behaviour of CFTR homologues (**A**) Representative single-channel recordings of human, ovine and murine CFTR in excised inside-out membrane patches from CHO cells expressing the indicated CFTR variants acquired using the experimental conditions described in Ref [43]. The closed-channel state (C), the subconductance state of murine CFTR (O_1) and the full-open state (human and ovine CFTR, O; murine, O_2) are indicated by dotted lines. Traces on the left were filtered at 500 Hz, whereas the 2-s portions indicated by bars shown on an expanded time scale to the right were filtered at 50 Hz. (**B**, **C**) Single-channel current amplitude (*i*) and open probability (P_o) of human (h), ovine (o) and murine (m) CFTR for the experimental conditions described in Ref [43], except murine CFTR, which was studied with ATP (0.3 mM) in the intracellular solution. Data are means ± SEM (human, $n = 10$, ovine, $n = 24$, murine, $n = 5$); *, $P < 0.05$ vs. human CFTR. Data were originally published in Ref [55], copyright (2007) National Academy of Sciences USA and Refs [43,46]. (**D**) Relationship between the single-channel conductance of CFTR and mean body temperature in endothermic vertebrates. Values of mean body temperature are from Refs [60-62] and single-channel conductance from Refs [38-44,52]. The continuous line is the fit of a first-order regression to the data ($r^2 = 0.51$).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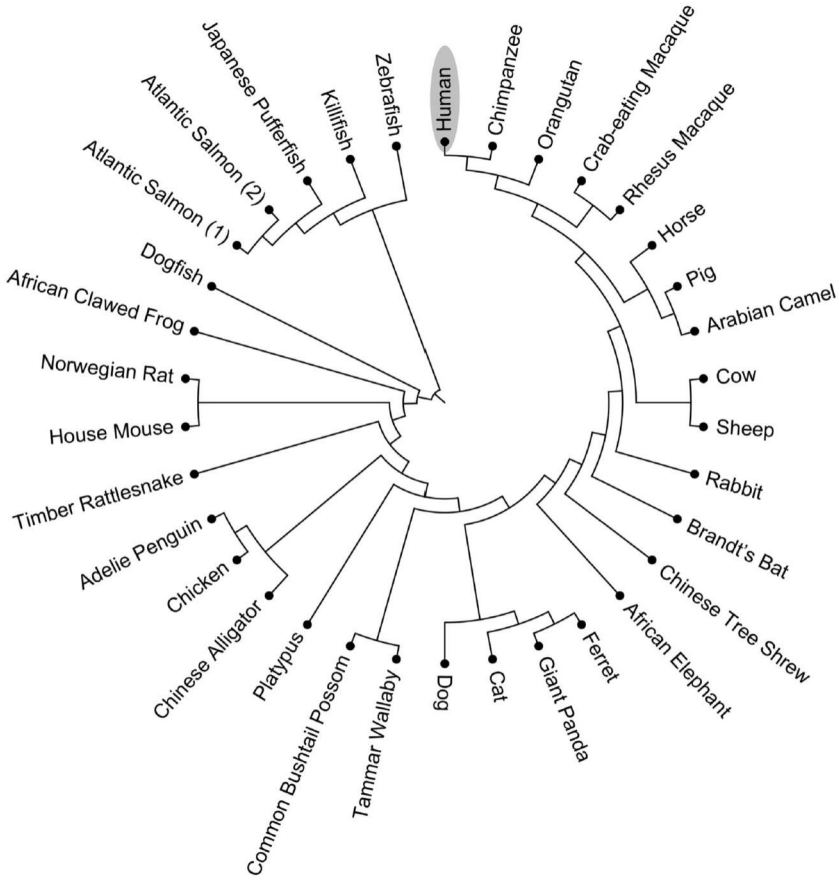
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African Elephant
Ferret
Giant Panda
Cat
Dog
Tammar Wallaby
Common Bushtail Possom
Platypus
Chinese Alligator
Chicken
Adelie Penguin
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Killifish
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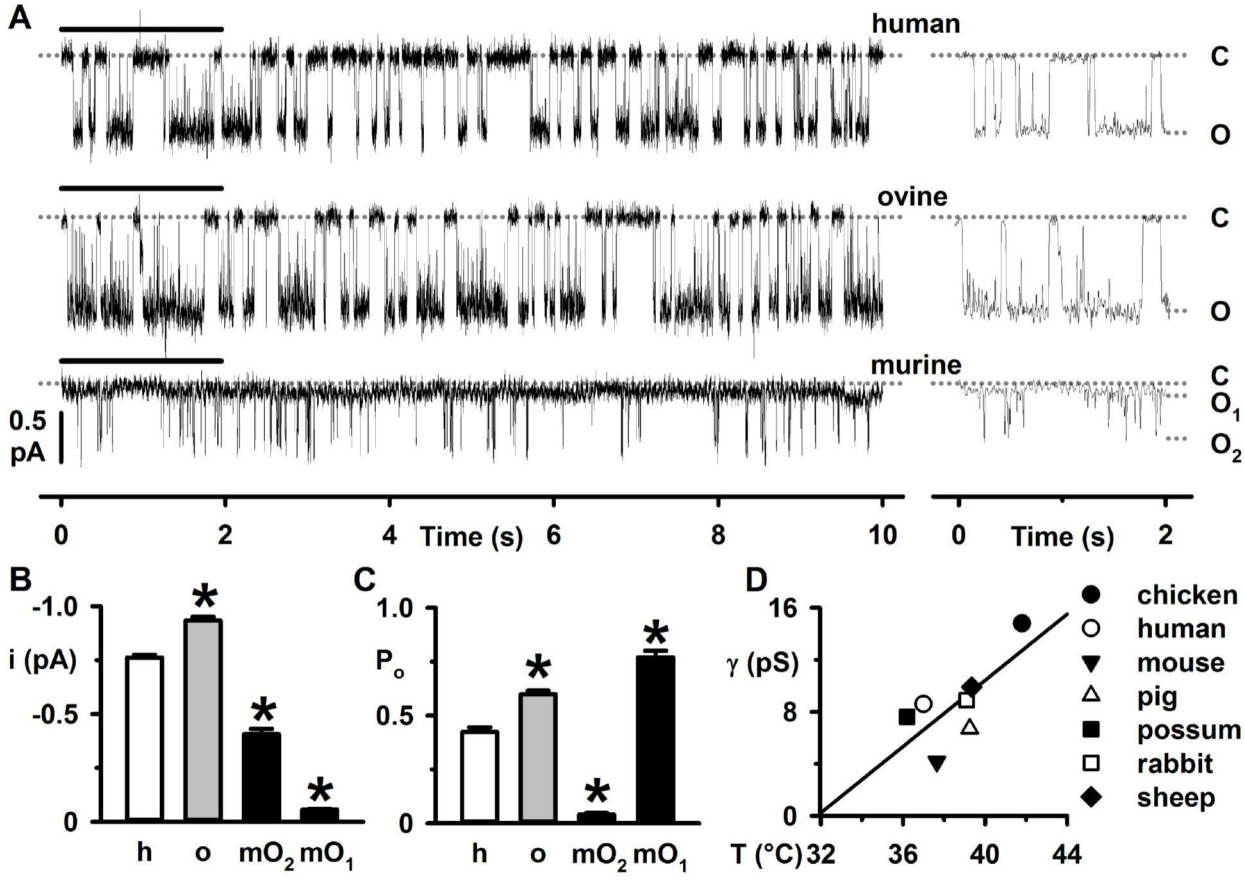
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